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Analytical Methods

Infrared spectroscopy as a rapid tool to detect methylglyoxal and antibacterial activity in Australian honeys

Yasmina Sultanbawa [a,*], Daniel Cozzolino [b], Steve Fuller [c], Andrew Cusack [c], Margaret Currie [c], Heather Smyth [a]

[a] Centre for Nutrition and Food Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Australia
[b] School of Agriculture, Food and Wine, The University of Adelaide, Australia
[c] Innovative Food Solutions and Technology, Department of Agriculture, Fisheries and Forestry, Queensland, Australia

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ABSTRACT

Methylglyoxal (2-oxopropanal) is a compound known to contribute to the non-peroxide antimicrobial activity of honeys. The feasibility of using infrared spectroscopy as a predictive tool for honey antibacterial activity and methylglyoxal content was assessed. A linear relationship was found between methylglyoxal content (279–1755 mg/kg) in *Leptospermum polygalifolium* honeys and bacterial inhibition for *Escherichia coli* (R^2 = 0.80) and *Staphylococcus aureus* (R^2 = 0.64). A good prediction of methylglyoxal (R^2 0.75) content in honey was achieved using spectroscopic data from the mid infrared (MIR) range in combination with partial least squares regression. These results indicate that robust predictive equations could be developed using MIR for commercial application where the prediction of bacterial inhibition is needed to 'value' honeys with methylglyoxal contents in excess of 200 mg/kg.

1. Introduction

Honey is valued for its therapeutic properties and is increasingly used as a topical antibacterial agent for the treatment of surface wound infections (Allen, Molan, & Reid, 1991; Carr, 1998). The antimicrobial component of honey has been attributed to the acidity, high osmolarity and hydrogen peroxide (White, Subers, & Schepartz, 1963). More recently, certain honeys from *Leptospermum* spp. and other Australian and New Zealand native plants have been identified for their non-peroxide antimicrobial activity due to the presence of plant derived compounds (Allen et al., 1991). The *Leptospermum* honeys are well known for their healing properties (Thomas, Hamdan, Hailes, & Walker, 2011; Zerm et al., 2010) and can be grouped as New Zealand manuka honey from *Leptsopermum scoparium* and the Australian jelly bush honey from *Leptospermum polygalifolium*.

2-Oxopropanal, more commonly known as methylgloxal, has been identified and reported as one of the key compounds that contribute to the non-peroxide antimicrobial activity in New Zealand manuka honey (Adams, Manley-Harris, & Molan, 2009; Adams et al., 2008; Mavric, Wittmann, Barth, & Henle, 2008). The high levels of methylglyoxal are formed by conversion of dihydroxyacetone, present at substantial concentrations in the nectar of the *Leptospermum* spp. flowers (Adams et al., 2009). It is understood that methylglyoxal in manuka honey has a strong positive correlation with antibacterial activity of *Staphylococcus aureus* (Atrott & Henle, 2009) and, indeed, methylglyoxal content has been used commercially in labelling as an indicator of the bioactivity of *Leptospermum* honeys. Further, methylglyoxal content has been used by industry as a marker for quality or 'honey value' and apiarists have been paid according to the methylglyoxal content measured in their honey as consumed. Currently, methylglyoxal content is measured using a high pressure liquid chromatography (HPLC) method that involves an initial derivatisation, using orthophenylenediamine, of methylglyoxal to the corresponding quinoxaline (Mavric et al., 2008). The method is considered robust, however it is time consuming, involves a number of steps and numerous reagents and solvents. Honey samples must be collected from remote locations and sent for the laboratory-based testing, which means days pass before business decisions can be made about the purchase of certain honeys. Commercially, methylglyoxal content is expressed as an 'MGO number', for example, '500 + MGO' or '900 + MGO' indicates minimum methylglyoxal concentrations of 500 mg/kg or 900 mg/kg, respectively.

Another method used to measure honey 'value', used particularly for manuka honeys, is classification according to a microbiological assay. The assay involves the measurement of the

* Corresponding author. Tel.: +61 7 32766037; fax: +61 7 32169591.
E-mail address: y.sultanbawa@uq.edu.au (Y. Sultanbawa).

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antibacterial activity of the non-peroxide component of manuka honey and expressed as a phenol equivalent scale on a standardised antibacterial agar diffusion assay using *S. aureus* as the control test organism (Allen et al., 1991; Stephens, 2006). The classification unit expressed for commercial labelling using the microbiological assay method is called the "Unique Manuka Factor" (UMF®). Therefore, manuka honey with a higher UMF® value has a greater antibacterial activity. UMF® 12+ manuka honey is marketed for therapeutic usage. However, the UMF® 16+ would be more potent for medical applications (Stephens, 2006). UMF is not directly based on methylglyoxal content, but is indirectly linked by the relationship between microbiological activity and honey methylglyoxal content. Unfortunately for the consumer, the UMF and MGO factors cannot be compared directly to appreciate the value of honey's bioactivity.

Currently, there is no single method recognised across the honey industry that is accepted as the 'standard' for assessment of honey bioactivity and resulting commercial value. The honey industry is in great need of an objective, rapid, inexpensive, potentially portable and readily accessible tool to detect the quality and value of bioactive honey. Such a tool would undoubtedly move the industry toward accepting and adopting a recognised standard method, across the board, where apiarists are paid fairly according to a single scale of value and consumers are able to assess products directly with one another and make decisions accordingly.

In recent years, the development of sampling accessories attached to a wide range of infrared (IR) spectrophotometers, such as attenuated total reflectance (ATR) cells, has led to major improvements in routine analysis. The ATR sample presentation relies on a sample absorbing the incoming infrared radiation by internal reflection from the surface of a high-refractive index transparent medium. The ATR absorption mechanism is due to two phenomena: a rapid change in sample refractive index across an infrared absorption peak, and the molar absorption coefficient attributed to the vibrational mode. The change in refractive index can cause severe peak distortions in the mid infrared (MIR) spectrum, particularly if the incident angle is near the critical angle, but such distortions are usually small at wider angles, close to 45°, used in most ATR accessories.

The application of Fourier transformed mid-infrared (FT-MIR) spectroscopy to quantify sugars and other compositional parameters in foods and beverages, including honey, has been reported by several authors (Bertelli, Plessi, Sabatini, Lolli, & Grillenzoni, 2007; Cozzolino, Cynkar, Shah, & Smith, 2011; Cozzolino, Holdstock, Dambergs, Cynkar, & Smith, 2009; Etzold & Lichtenberg-Kraag, 2008; Kelly, Downey, & Fouratier, 2004; Sinelli, Cerretani, di Egidio, Bendini, & Casiraghi, 2010).

This study aimed to assess the potential of using mid-infrared (MIR) spectroscopy as a tool to predict antibacterial activity and methylglyoxal content in honey samples.

2. Materials and methods

2.1. Honey samples

Honeys from different floral sources of Australian native plants were used for this study. These included Heath (*Banksia ericifolia*), Yellow box (*Eucalyptus mellidora*), Iron bark (*Eucalyptus crebra*), Juniper (*Leptospermum juniperinum*) and Jelly bush (*L. polygalifolium*). The honeys were also from a range of production years (2004–2009). There were 109 samples of honey provided by Medi bioactive Honey Australia Pty Limited, Northcote, Victoria, Australia.

2.2. Chemical analysis of methylglyoxal content in the honey

The honey samples (109 in total) were tested for methylglyoxal content as follows. Methylglyoxal was measured as the corresponding quinoxaline after derivatisation with orthophenylenediamine (Weigel, Opitz, & Henle, 2004) using HPLC and UV-detection as described by Mavric et al., 2008. A volume of 1.0 ml of either a honey solution (15% w/v of honey in 0.5 mol/l sodium phosphate, pH 6.5) or a methylglyoxal standard solution, were mixed with 0.6 mL of an orthophenylenediamine solution (1.0% w/v in 0.5 mol/l sodium phosphate buffer, pH 6.5). After 12 h in darkness at room temperature, the resulting mixture was filtered (0.45 μm) and 20 μL was used for chromatography analysis. HPLC was performed using a Shimadzu System with a pump LC-10AT, detector diode array SPD-M10A VP and an auto sampler SIL-10AD VP (Shimadzu Corporation Analytical Instruments Division, Kyoto, Japan). A reversed phase stainless steel column, 250 mm × 4.0 mm, Bio-Rad Bio-Sil ODS-5S (Bio-Rad Laboratories, California, USA), was used with a flow rate of 0.8 mL/min. The column temperature was set at 30 °C. The mobile phases were 0.15% acetic acid (solvent A) and 80% methanol containing 20% solvent A (solvent B). The gradient began with 20% solvent B over 2 min, increased linearly to 40% solvent B over 20 min, to 100% solvent B over 15 min, followed by an elution with 100% solvent B over 5 min, and then changed to 20% solvent B over 7 min with subsequently equilibration with 20% solvent B for 5 min. Peaks were detected by measurement of UV-absorbance at 312 nm. External calibration was performed in the range from 9.33 to 597 mg/kg for methylglyoxal and the calibration curve showed linearity throughout the concentration range. According to Mavric et al. (2008), the limit of detection for this method is 0.2 mg/kg.

2.3. Antibacterial analysis of the L. polygalifolium honeys

The jelly bush honeys (35 samples) with methylglyoxal levels greater than 200 mg/kg were tested for antibacterial activity against *S. aureus* and *Escherichia coli*.

The method used a 96 well plate to measure optical density at 620 nm. *E. coli* 9001 (NCTC – National Collection of Type Cultures, Health Protection Agency Centre for Infection, London, UK) and *S. aureus* 6751 (NCTC) bacterial strains were grown in nutrient broth and incubated at 37 °C. Honey was diluted in nutrient broth with a starting dilution of 12.5% w/w, further dilutions of 4:5 were made with lowest dilution of 1.1%. Each test well contained 190 μL of honey solution and 10 μL of culture solution, and each sample was tested in triplicate. A standard methylglyoxal solution (Sigma–Aldrich Chemical Co., New South Wales, Australia) in 40% water was tested against *E. coli* and *S. aureus* at concentrations ranging from 29 to 127 (mg/kg). Each test well contained 190 μL of methylglyoxal solution and 10 μL of culture solution; each sample was tested in triplicate.

Analysis of results involved percent Inhibition = (1 − (OD test well/OD of corresponding positive control well)) × 100.

$$\text{Percent Inhibition} = (1 - (T_{22} - T_0)/(C_{22} - C_0)) \times 100$$

The OD for each replicate at time zero (positive control (C_0) and test well (T_0)) was subtracted from the OD for each replicate at 22 h (positive control (C_{22}) and test well (T_{22})). The method was adapted from Patton, Barrett, Brennan, and Moran (2006).

2.4. Spectroscopic analysis of honey

Honey samples were scanned as collected (no pre-processing) at room temperature using a platinum diamond ATR single reflection sampling module cell mounted in a Bruker Alpha instrument (Bruker Optics GmbH, Ettlingen, Germany). Samples were scanned

in a MIR instrument using a platinum diamond ATR single reflection sampling module cell mounted in a Bruker Alpha instrument (Bruker Optics GmbH, Ettlingen, Germany). The samples were held against the ATR crystal using the pressure applicator or sample clamp mechanism supplied by the instrument manufacturer in order to ensure that the same and constant pressure was applied for all samples. Air was used as reference background spectra. The ATR diamond surface was cleaned with water before each sample was scanned. The MIR spectra were recorded on OPUS software version 6.5 provided by Bruker Optics. The spectrum of each sample was obtained by taking the average of 64 scans at a resolution of 4 cm^{-1} and acquired between 4000 and 375 cm^{-1}, with a scanner velocity of 7.5 kHz and a background of 64 scans. The reference background spectra were recorded using air. Water was used to clean the ATR cell to avoid carry over between samples and dried using disposable lab wipes (Cozzolino et al., 2011; Shah, Cynkar, Smith, & Cozzolino, 2010).

2.5. Data analysis

Statistical analyses of the honey chemical data were carried out using JMP (release 6.0, SAS Institute Inc., Cary, NC) to examine distribution of honey methylglyoxal content, and to determine any relationship between honey methylglyoxal content and the amount of honey required for antimicrobial activity.

The spectra of the honey samples were exported from the OPUS software in csv format into The Unscrambler X software (CAMO ASA, Oslo, Norway) for chemometric analysis. Principal component analysis (PCA) was performed before partial least squares regression (PLS1) models were developed to determine any relevant and interpretable structure in the data, and to detect outliers. Five honey samples with relatively low concentration of methylglyoxal were under predicted (negative values) and were considered as outliers and removed for modelling. Calibration models between honey methylglyoxal compositional values and MIR spectra were developed using the PLS1 regression method with full cross validation. The optimum numbers of terms in the PLS1 calibration models were indicated by the lowest number of factors that gave the minimum value of the prediction residual error sum of squares (PRESS) in cross validation in order to avoid over fitting in the models. Statistics calculated for the calibrations included the coefficient of determination in cross-validation (R^2), the standard error of cross-validation (SECV), bias and slope. The prediction accuracy was tested by full cross-validation using the SECV and the residual predictive deviation (RPD = SD/SECV). Statistics calculated included the coefficient of correlation (R), slope and bias (Naes, Isaksson, Fearn, & Davies, 2002).

3. Results and discussion

3.1. Chemical analysis of honey methylglyoxal content

Fig. 1 shows the distribution of honey samples in terms of methylglyoxal content across the 109 honeys from varying floral sources. The honey methylglyoxal content ranged from 8 mg/kg to 1755 mg/kg with an average concentration of 357 mg/kg. The coefficient of variation (cv) of the HPLC analysis of methylglyoxal in 109 honeys was typically less than 2%. Most of the honey samples were in the lower range in terms of methylglyoxal content and these, typically, included honeys from a heath or ironbark floral source. By comparison, the honeys from a *L. polygalifolium* (jelly bush) floral source were found to have higher concentrations and ranged in methylglyoxal content from 279 mg/kg to 1755 mg/kg with an average concentration of 604 mg/kg. Fig. 1 also shows that

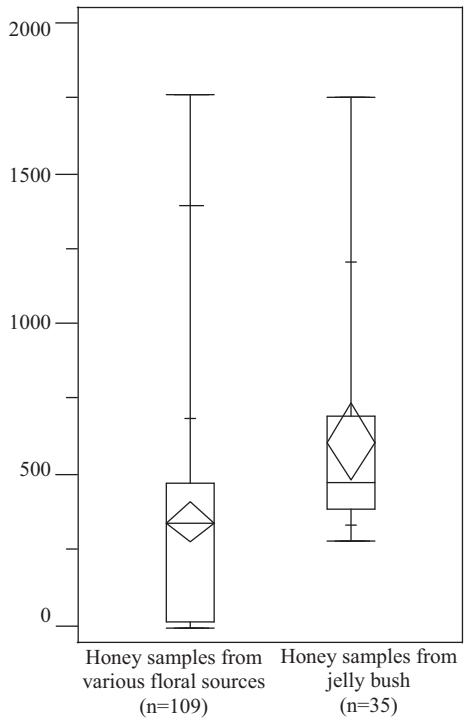

Fig. 1. Distribution of methylglyoxal content (mg/kg) measured in honey samples from various floral sources (n = 109) and from *Leptospermum polygalifolium* (jelly bush) (n = 35) (data presented as a quantile box plot).

distribution of the jelly bush honeys (n = 35) in terms of methylglyoxal content.

Given the greater concentrations and broader, more-evenly distributed, range of honey methylglyoxal content in the jelly bush honeys, and to minimise the chemical variation as a result of the different floral sources, only jelly bush honeys (35 in total) were selected and tested for antibacterial activity and included in modelling using MIR spectroscopy. Moreover, *Leptospermum* honeys are of greater interest to study in terms of microbiological activity as they have been shown to be one of the most reliable medically-active Australian honeys (Carter, Blair, & Irish, 2010).

3.2. Antimicrobial analysis of methylglyoxal and honey

Standard addition calibrations were developed for the antibacterial activity of a methylglyoxal standard against antimicrobial activity for *E. coli* and *S. aureus* and these are shown in Fig. 2. Both *E. coli* and *S. aureus* were completely inhibited at a concentration of 89 mg/kg (1.1 mM). This is in agreement with that reported by Mavric et al. (2008) who showed minimum concentrations of 1.1 mmol/l methylglyoxal achieved complete inhibition of *E. coli* and *S. aureus*.

Fig. 3 shows the amount of honey required for each of the jelly bush honeys (n = 35) to achieve complete inhibition of (A) *S. aureus*, and (B) *E. coli*, plotted against the methylglyoxal content in each honey. An inverse linear relationship was found between the two demonstrating that methylglyoxal is an important contributor to antibacterial activity in these honeys. This is in agreement with Atrott and Henle (2009) who reported a good linear correlation between antibacterial activity for *S. aureus* and honey methylglyoxal content in the range of 189–835 mg/kg for manuka honey. Indeed, this author recommended the use of methylglyoxal as a tool for the labelling of the bioactivity of commercial honey products.

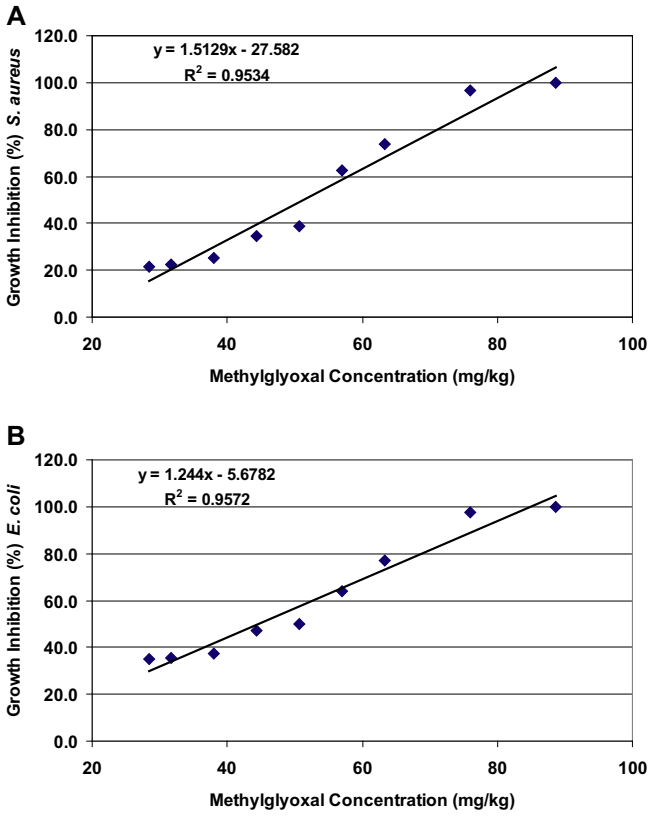

Fig. 2. Standard curves for methylglyoxal vs. % Growth Inhibition of: (A) *Staphylococcus aureus*, and (B) *Escherichia coli*.

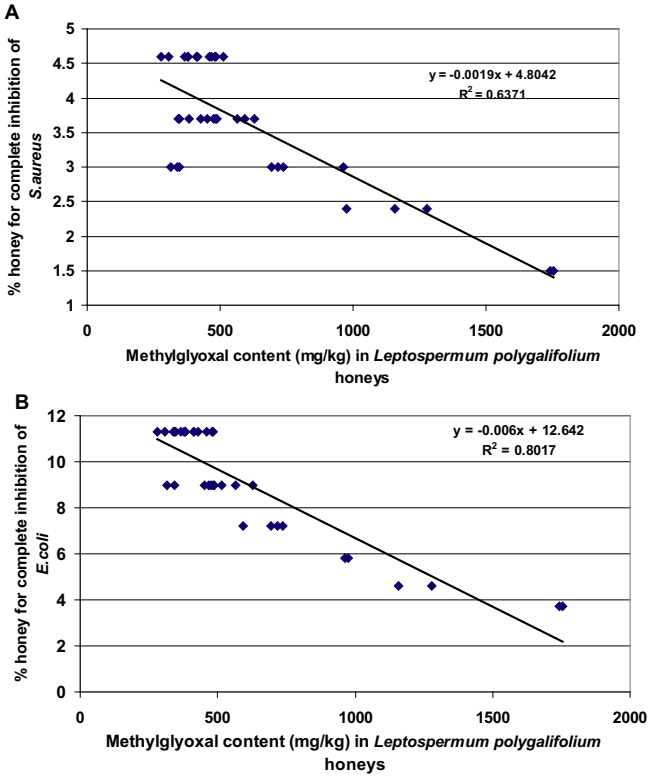

Fig. 3. Antibacterial activity of *Leptospermum polygalifolium* honeys ($n = 35$) with different levels of methylglyoxal (mg/kg) against: (A) *Staphylococcus aureus*, and (B) *Escherichia coli*.

In the jelly bush honeys, it was observed that a higher honey methylglyoxal concentration was required to inactivate *E. coli* than *S. aureus*. An equivalent methylglyoxal concentration of 23–55 mg/kg and 8–24 mg/kg was required to completely inactivate the *E. coli* and *S. aureus*, respectively. This is much less than the 89 mg/kg required of neat methylglyoxal standard to achieve the same inhibition. This indicates that there are other components and phytochemicals that are contributing to the antibacterial activity of honey. In recent times, the knowledge on the antibacterial compounds in *Leptospermum* honeys has increased, however there are other antibacterial compounds involved that need to be identified to facilitate the use of honey in medicine (Kwakman & Zaat, 2012). A study by Kwakman, Velde, de Boer, Vandenbroucke-Grauls, and Zaat (2011) reported on the neutralisation of methylglyoxal in manuka honey by a quantitative bactericidal assay and confirmed that methylglyoxal is the major component against *S. aureus* but additional compounds contributed to the activity against *Bacillus subtilise*, *E. coli* and *Pseudomonas aeuruginosa*. This would explain the greater methylglyoxal concentration required to inactivate *E. coli* in the present work. Kato et al. (2012) isolated and characterised a novel glycoside of methyl syringate and named it "leptosin" after the genus *Leptospermum*. The concentration of leptosin was positively correlated to *S. aureus* activity. This glycoside was only found in *Leptospermum* honeys. It has been shown by Adams et al. (2008) that honeys with lower methylglyoxal content do not have a similar relationship with antimicrobial activity as honeys with higher antimicrobial activity. Certainly, data from our laboratory suggests only honeys with >200 mg/kg methylglyoxal follow a linear correlation between methylglyoxal content and bacterial inhibition (data not reported). Adams et al. (2008) reported a linear correlation between antibacterial activity and concentrations of methylglyoxal at higher levels of activity, and that extrapolation to zero left a substantial amount of activity unaccounted for. It is clear that in honeys with low methylglyoxal content (<200 mg/kg methylglyoxal), there are other components and phytochemicals contributing significantly to antimicrobial activity. While further research is needed to determine the nature and identity of these unknown active components, it remains clear that for *Leptospermum* honeys with methylglyoxal content in excess of 200 mg/kg a rapid method to assess methylglyoxal is needed for industry application.

3.3. Spectroscopic analysis of honey

Predictive models were developed using partial least squares (PLS) regression whereby the spectroscopic information was used to predict both the concentration of methylglyoxal in honey and the amount of honey required to inhibit each bacteria (*E. coli* and *S. aureus*) ($n = 30$). The results of the predictive models are given in Table 1 and the predictive model for methylglyoxal content is shown graphically in Fig. 4. A good correlation was achieved for honey methylglyoxal content (R^2 0.75), indicating that MIR spectroscopy shows promise as a technique to develop for commercial application specifically for methylglyoxal testing in honey. Calibration between chemical analysis and MIR spectroscopy was evaluated. The ratio of standard deviation and standard error in cross validation (RDP) was used to test the accuracy of the calibration models. An RPD > 3 is considered adequate for analytical purposes in most spectroscopic applications for agricultural products (Fearn, 2002; Williams, 2001). In this study an RDP of 1.68 was achieved which indicates, not surprisingly, that this preliminary model requires more development before it can be applied for industrial purposes. The R^2 indicated that 75 per cent of the total variation can be explained by the PLS method. The addition of more samples

Table 1
Summary of predictive model performance using MIR spectra to predict: methylglyoxal content of honey; amount of honey required to inhibit *Staphylococcus aureus*; and amount of honey required to inhibit *Escherichia coli*.

	R^2	SECV	RDP	Samples (n)
Prediction of methylglyoxal content of honey in jelly bush honeys (>200 mg/kg)	0.75	225	1.8	30
Prediction of amount of honey required to inhibit *Staphylococcus aureus*	0.73	1.03	–	35
Prediction of amount of honey required to inhibit *Escherichia coli*	0.63	1.7	–	35

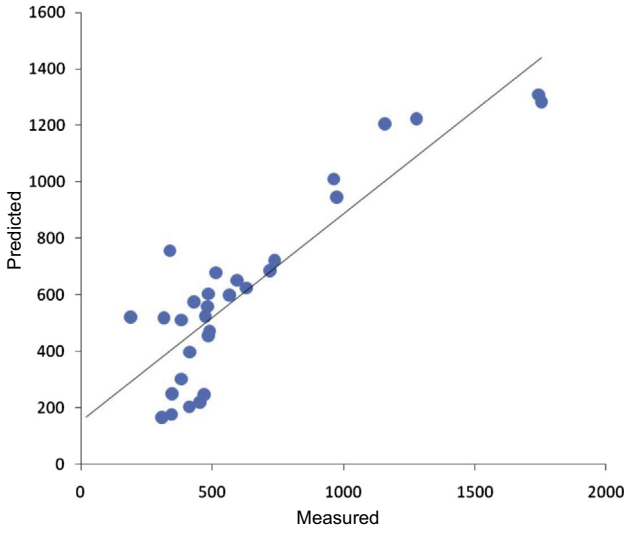

Fig. 4. Results of PLS regression using MIR spectroscopic data to predict methylglyoxal content of *Leptospermum polygalifolium* (jelly bush) honey (n = 30[a]). [a]Of the 35 honeys tested, 5 samples were considered outliers and removed from the modelling.

with a wide range in composition will improve the calibration statistics. However, the main objective of this work was to test the ability of PLS method and not to develop robust calibrations for this compound. The same apply for the other correlations.

Acceptable predictive models were also achieved using MIR spectra to predict the amount of honey required for the inhibition of *S. aureus* (R^2 0.73) and *E. coli* (R^2 0.63) using the 35 jelly bush honey samples. These results indicate that, with the addition of more samples with a broad range of methylglyoxal, robust predictive equations could be developed indeed for the prediction of bacterial inhibition qualities of honeys where the methylglyoxal content is in excess 200 mg/kg.

4. Conclusions

An excellent relationship can be found between methylglyoxal content and antibacterial activity for *L. polygalifolium* (jelly bush) honey with methylglyoxal contents in the range of 200–1750 mg/kg. The spectroscopic models suggest that MIR spectroscopy could be developed as a valuable predictive tool for determining both methylglyoxal content of jelly bush honey and the potential for a honey to inhibit specific organisms (*E. coli* and *S. aureus*). If developed commercially, this technology could be a convenient replacement for conventional, time-consuming laboratory-based methylglyoxal and antimicrobial testing methods. The proposed method may also provide a way forward for industry to adopt a universal method for testing quality and labelling for the benefit of apiarists, processors and consumers alike.

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